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Macromolecular transport across the superficial layer of articular cartilage

M. Uesugi and H. E. Jasin
The Theresa Scheu Rheumatoid Arthritis Research Laboratory, Division of Rheumatology and Clinical Immunology, Department of Internal Medicine, The University of Arkansas for Medical Sciences, and John L. McClellan Veterans Administration Medical Center, Little Rock, Arkansas, USA

Summary

Objective: To study the role of the superficial layer of articular cartilage in the transport of macromolecular solutes.

Design: The articular cartilage of intact bovine carpal bones was incubated with ^{125}I-labeled bovine serum albumin, human IgG, or horse ferritin for 4 hours. Quadruplicate samples were first incubated with polymorphonuclear neutrophil elastase for 30 minutes to remove the outermost layer covering the articular surface. The rates of exchange of each macromolecule from excised tissue explants in the absence of a concentration gradient were measured at six different time points. The results were expressed as the fraction of radioactive protein exiting the cartilage per mm^2 of tissue, or as picomoles of labeled solute per mm^2.

Results: Exchange rates correlated well with molecular mass, and no apparent differences were detected between intact and elastase-treated tissues. However, when the results were expressed in terms of the total number of molecules within the tissue, it was apparent that IgG molecules accumulated in the intact cartilage in larger than expected numbers. This finding was not observed in experiments using elastase-treated tissue.

Conclusion: These observations suggest that the outermost surface layer does not constitute a barrier to the transport of macromolecules into the deeper zones of the tissue. The higher IgG accumulation observed in intact cartilage suggests that the acidic outer layer of cartilage exhibited attractive interactions, probably ionic in nature, with the cationic fraction of IgG. These observations may relate to our previous work demonstrating that the sequestered immune complexes in the superficial zone of articular cartilage in rheumatoid arthritis, and in the antigen-induced arthritis model, are formed because pre-existing antibody normally present in cartilage irreversibly traps antigen within the tissue. © 2000 OsteoArthritis Research Society International

Key words: Articular cartilage, Macromolecular transport, Cartilage surface.

Introduction

The outermost layer of the articular cartilage is a complex structure composed of several macromolecular species,[1] particularly small, non-aggregating proteoglycans organized in three dimensions,[2] presumably evolved to mediate specific functions necessary for the normal physiologic integrity of a tissue subjected to a variety of protracted mechanical insults throughout the lifespan of the individual. The precise role of this superficial layer in physiology is not well understood. Studies from our laboratory have indicated that the non-aggregating macromolecules on the cartilage surface mask immunogenic collagen type II epitopes so that antibodies to the main structural cartilage component are unable to bind in large amounts to the intact articular surface.[3,4] It was also shown that the macromolecules responsible for the masking of collagen epitopes are inordinately susceptible to digestion by serine proteases derived from inflammatory cells.[3,4] Moreover, recent work has shown that these macromolecules are powerful inhibitors of fibroblast[2] and polymorphonuclear neutrophil (PMN)[5] adhesion to the articular surface, providing an explanation for the appearance of invasive pannus in chronic inflammatory arthritides.

The articular cartilage is an avascular and alymphatic tissue so that the resident cells depend on diffusion of soluble nutrients from the joint cavity and juxta articular bone for their survival and for their role in maintaining the homeostatic integrity of the tissue. For this reason, the macromolecular transport properties of cartilage have been the focus of particular attention. In the studies by Maroudas[6] dealing with macromolecular diffusion, the superficial layer of cartilage was discarded. The present studies aimed at delineating the role of the superficial layer in the transport of macromolecular solutes into the deeper zones of cartilage have taken advantage of our previous observation that a thirty-minute incubation of the articular surface with PMN elastase removed much of this layer, resulting in maximal exposure of the underlying collagen type II epitopes to interact with antibody without depleting the underlying proteoglycans.[3,4] These observations, and additional studies using human cartilage specimens from patients with rheumatoid arthritis and osteoarthritis[7] suggested that the above-mentioned treatment resulted in almost complete denudation of the surface layer. The results presented here suggest that the superficial layer of cartilage does not constitute a barrier to the transport of

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Address correspondence to: Hugo E. Jasin, M.D., Division of Rheumatology, Mail Slot 509, UAMS. 4301 West Markham, Little Rock, AR 72205, USA.
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macromolecular solutes of up to 440 kD into the deeper zones. Moreover, the studies with intact cartilage indicate that the surface macromolecules may have increased affinity for IgG, increasing its accumulation over and above that expected for the molecular mass of this protein.

Materials and methods

MATERIALS

Crystalline bovine serum albumin (BSA), 68 kD, pI: 4.7 and horse ferritin, 440 kD, pI: 4.4, were purchased from Sigma (St Louis, MO). Human gamma globulin (Cohn fraction II) 155 kD, pIs: 5.8–7.9, was purchased from Miles Scientific (Naperville, IL). Human elastase was obtained from Elastin Products (Owensville, MO).

IODINATION OF TRACER PROTEINS

Tracer proteins were labeled with ^{125}I using IODOBEADS (Pierce Chemical Co., Rockford, IL). After exhaustive dialysis, gel filtration chromatography was carried out with a 1.6×70 cm Sephacryl S-200HR (Pharmacia, Piscataway, NJ) column equilibrated with PBS-0.01% sodium azide to eliminate the remaining free NaI and fragmented proteins generated during the oxidation step.

TRANSPORT STUDIES

The studies were performed as described by Maroudas[6] with modifications aimed at exposing only the articular surfaces to the enzymatic attack and to the labeled macromolecule under study. Carpal bones from bovine carpometacarpal joints were excised and washed three times with large volumes of PBS. The whole intact bones were then immersed, cartilage down, in 3.6 cm diameter culture dishes containing 2 ml PBS with or without human PMN elastase 1.0 μg/ml, so that the solution reached an approximate height of 5.0 mm when the bone was immersed in the dish. The bones were incubated for 30 min at 37°C with agitation. The carpal bones were then washed with PBS-1 mM phenyl-methyl sulfonyl fluoride (PMSF)-10 mM EDTA for 20 min to inhibit protease activity. After three sequential washes with PBS, they bones were incubated in 3.6-cm diameter dishes with 2 ml of PBS containing 20.0 mg/ml of each study protein, with approximately 5.0×10^7 cpm/ml (specific activity: 2.5×10^6 per mg) radiolabeled tracer protein and 1 mg/ml NaI, for 4 h at 4°C with continuous shaking. After three 1-sec immersions in PBS followed by brief blotting with absorbent paper, approximately 4×4 mm full thickness square quadruplicate cartilage pieces were cut from the carpal bones with a scalpel blade, and they were sequentially desorbed with six successive and separate 1.0 ml washes of PBS containing 2% unlabeled protein and 1.0 mg/ml NaI at 37°C with continuous shaking. The washes were changed at 1, 5, 10, 60 and 840 minutes. Protein-bound iodine in the washes were measured after protein precipitation with 10% trichloroacetic acid solution (TCA). Radioactivities in the precipitate, supernatants, and tissue explants were measured with an autogamma scintillation spectrometer (COBRA-2; Packard, Downers Grove, IL). The sides of each cartilage piece were measured to determine their surface area. The results were expressed either as percent radioactivity that had diffused into the supernatants at each time-point per mm^2 of cartilage, where the aggregate of all the radioactive material in cartilage at zero time (cpm in all supernatants+cpm remaining in tissues corrected for the surface area of each explant) was taken as 100%; or as picomoles of labeled protein appearing in the supernatants per mm^2 of explant using the following equation: pmol/mm^2 in the supernatants=ng/mm^2/ng/pM.

As a rule, from 3000 to 4500 cpm (~2 μg) had penetrated each cartilage slice after the four hours incubation with the radioactive protein. Thus, the cpm in each supernatant were corrected for the size of each explant (~16 mm^2 or 250 cpm/mm^2), and converted to ng/mm^2 using the specific activity of the solution employed (~2.5 cpm/ng), then corrected for the MW of each protein (in the case of IgG: 150 ng/pM). The results represent the means of two to four separate experiments for each protein.

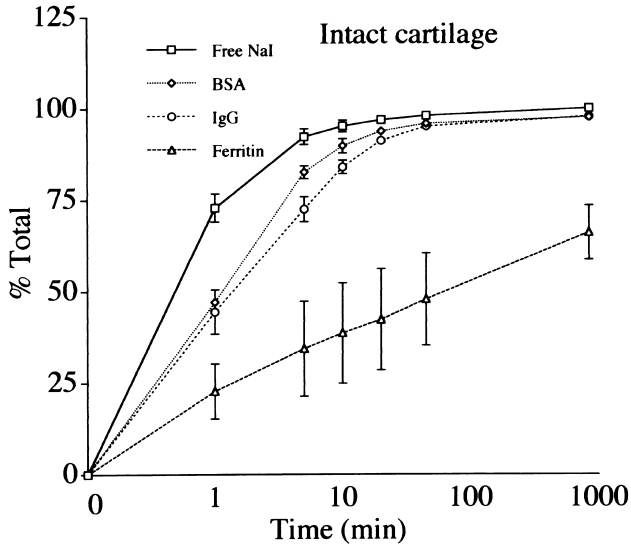

Fig. 1. Transport of free NaI, BSA, IgG, and ferritin in intact articular cartilage. The cartilage explants were desorbed at six different time points. The results were expressed as the cumulative percent of radioactive material appearing in the solutions, taking the total initial radioactivity within each tissue explant as 100%.

Results

In the intact cartilage (without treatment with elastase), IgG, BSA and TCA-soluble small radioactive material within the tissues appeared in the supernatants at about the same rates, and ferritin was the slowest to exchange, so that at the end of 14 hours, a significant proportion remained within the cartilage pieces (Fig. 1). Thus, transport appeared to correlate well with molecular mass, confirming the studies by Maroudas,[6] and suggesting that the outermost surface layer does not constitute a barrier to the movement of macromolecules into the deeper zones of the tissue. However, when the results were expressed in terms of the total number of molecules within the tissue, it was apparent that IgG molecules accumulated in the cartilage in larger than expected numbers (Fig. 2), that is, there were similar number of IgG molecules within the tissue compared to BSA, even though the former has a MW which is almost twice the MW of BSA. The number of molecules for the other two proteins correlated well with their respective molecular masses. Moreover, the rate of elution of IgG

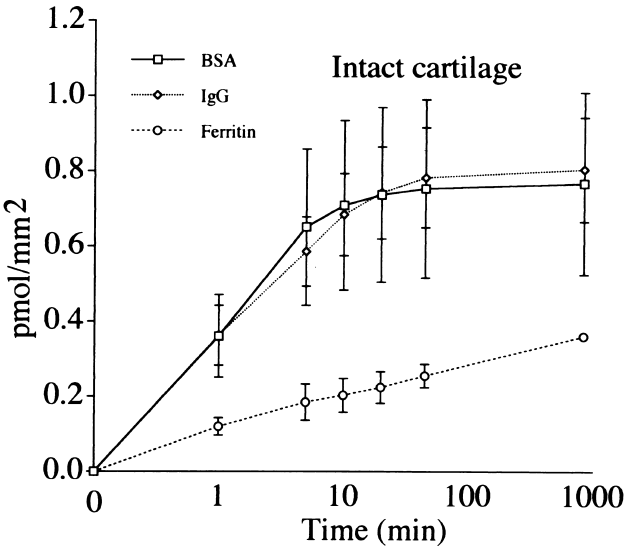

Fig. 2. Transport of BSA, IgG and ferritin in intact articular cartilage expressed as the cumulative number of molecules moving across the superficial layer with respect to time. The results in Fig. 1 were corrected by the molecular masses of each macromolecule and the specific activities of each labeled protein (~2.5 cpm/ng) as specified in the Methods section. NaI was excluded because the TCA-soluble radioactive material exhibited a wide range of molecular sizes, and its specific activity was unknown.

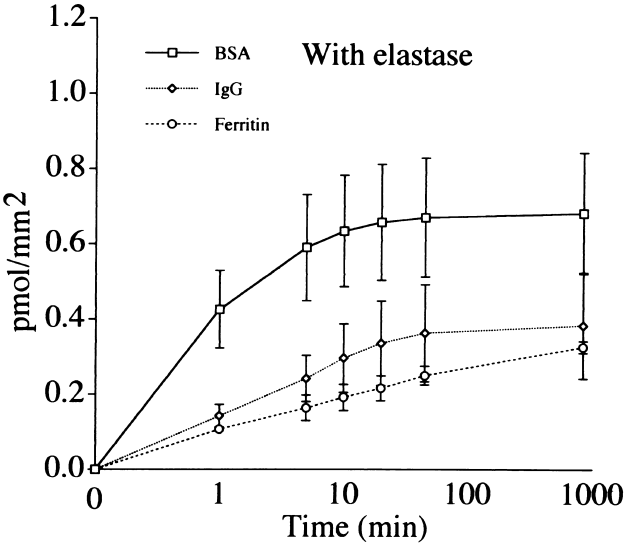

Fig. 3. Transport of BSA, IgG and ferritin in elastase-treated cartilage expressed as the cumulative number of molecules moving across the superficial layer with respect to time. The results in Fig. 4 were used for the calculations as described in Fig. 2.

approached that of BSA, suggesting that the excess molecules may have been loosely bound near the cartilage surface. The unexpected increase in IgG within the tissues was not seen in the experiments using elastase-treated cartilage (Fig. 3), and the differences between the intact and elastase-treated cartilage were statistically significant ($P<0.01$). It was apparent that in the absence of the superficial layer, the protein amounts and transport rates for each macromolecule used correlated well with their respective molecular masses. Similarly, when the rates of transport were expressed in percent of total protein in the

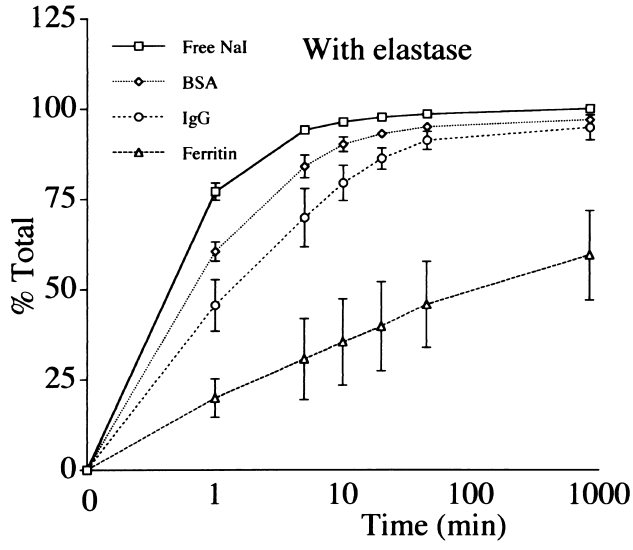

Fig. 4. Transport of free NaI, BSA, IgG, and ferritin in elastase-treated articular cartilage. The results were expressed as the cumulative percent of radioactive material appearing in the solutions, taking the total initial radioactivity within each tissue explant as 100%.

tissue exchanging with respect to time in elastase-treated tissues, the rates for the TCA-soluble small radioactive material, BSA and IgG were very similar to those seen in the intact cartilage experiments. Only the largest molecule, ferritin, exhibited an appreciably slower rate (Fig. 4). These results were very similar to those obtained for the intact tissue (Fig. 1) again indicating that the surface layer did not constitute a barrier for the movement of the molecules across it.

Discussion

Little is known about the properties and function of the superficial layer of cartilage.[8] Electron microscopy studies have shown a thin, irregular, electron dense layer containing anionic charges.[9] Studies by Stanescu *et al.*[10] indicate that is layer is resistant to extraction with high ionic strength reagents, hyaluronidase, chondroitinase ABC, or keratanase, but is partially disrupted by collagenase and removed by trypsin. Our own studies have shown that this layer contains small, non-aggregating proteoglycans, serum albumin,[1,3,4] and the minor collagen types V, VI and IX.[4] Additional studies have shown that the small proteoglycans on the articular surface are very susceptible to digestion by serine proteases derived from inflammatory cells,[4] and that the intact surface does not support PMN[5,11] or fibroblast cell adhesion.[2] The present studies were undertaken to investigate whether this structure constituted a barrier to the transport of large solutes into the deeper zones of this tissue.

By incubating the intact carpal bones, cartilage down, in a shallow layer (0.5 cm) of elastase or labeled macromolecule solutions we ensured that only the business surface of cartilage would be in contact with the reagents. Removal of the full thickness explants from the bone was undertaken after enough protein had penetrated the subsurface, thus excluding the artifactual influence of the explants' cut surfaces. In the work by Maroudas[6] equilibration of the diffusing molecules was essential since she was

interested in the permeability properties of the entire cartilage thickness. Those studies, had shown that the partition and diffusion coefficients of solutes were inversely correlated with their molecular size and the concentration of proteoglycans (fixed charge) at any given tissue depth. However, the partition studies reported used superficial tissue slices of 200–400 μm thickness, and in the studies dealing with the diffusion coefficients the superficial layer of cartilage was discarded. Thus, in our work, complete equilibration of the proteins diffusing into the tissue was not necessary since we were only interested in measuring the rate of exchange of the proteins in the presence or absence of the outermost layer. The results obtained indicate that the superficial layer of cartilage exhibits a 'pore' size large enough to allow free entrance of molecules as large as 440 kD and that their transport rates correlate well with their respective molecular sizes. This finding is in agreement with older studies indicating that IgG may be found in small amounts in normal cartilage.[12,13] The superficial zone of this tissue is known to have a low concentration of acidic proteoglycans,[14] thus allowing penetration of large molecules from the synovial fluid.

It is noteworthy that the greater accumulation of IgG molecules observed in the experiments using intact cartilage was higher than that of a smaller molecule such as BSA. Maroudas[6] had already observed a similar phenomenon for IgG, but no explanation was apparent from that work. The fact that this artifact was abolished by PMN elastase treatment of the tissue suggests that the acidic outer layer of cartilage exhibits attractive interactions, probably ionic in nature, with the cationic fraction of IgG which has isoelectric points ranging from 5.8 to about 8.0. Indeed, van Lent *et al*. showed that cationic proteins with pIs greater than 7.0, and molecular masses larger than 40 kD bound to articular cartilage and were retained in the joint for many days.[15] Using cartilage homogenates, Alomari *et al*. have shown that IgG would bind to an unspecified cartilage component.[16] The biologic significance of these observations is not clear, but it may have some bearing to our previous work demonstrating that the sequestered immune complexes in the superficial zone of articular cartilage in rheumatoid arthritis[17] and in the antigen-induced arthritis rabbit model,[18] are formed because preexisting antibody normally present in cartilage irreversibly traps antigen within the tissue.[19]

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